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Award Number: DAMD17-01-1-0068

TITLE: Studies of Prostate Tumor Development Via CRE/LOXP Technology

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REPORT DATE: May 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2005	Final	15 Apr 01 – 14 Apr 05

4. TITLE AND SUBTITLE
Studies of Prostate Tumor Development Via CRE/LOXP Technology

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0068

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas
Houston TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Although considerable progress in the understanding of prostate cancer has been made in the last few years, the basic knowledge of the biology of this disease remains elusive. The development of this cancer is related to the male sexual hormone (testosterone) but the actual mechanisms by which testosterone affects the development of this cancer is not known. The prostate gland has at least three different types of cells that contribute to the physiology of the gland: basal, luminal and neuroendocrine cells. It is not totally clear what the relationship is between these different cell types, how testosterone affects them and which one is the target cell in prostate cancer development. We will use new transgenic technology that allows tagging of a particular cell population and following its behavior over the life of the animal. These experiments will be performed in mice because this technology is well developed in these animals and there is a basic knowledge of the rodent prostate. The studies proposed here will clarify some of the basic aspects of the biology of the prostate gland and the process of carcinogenesis in this organ.

15. SUBJECT TERMS
Cre/LoxP technology, prostate cancer, cell lineages, transgenic mice

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	7	**19b. TELEPHONE NUMBER** *(include area code)*

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RESPONSE TO REVIEWER

I do not agree with the opinion of the reviewer stating that the report does not meet USAAMRMC. The material presented covered the whole period of the grant. Maybe the reviewer is being polite but it is obvious that the project was a failure. I made it quite clear when I said that Specific Aim 2 and 3 where not carried out. Those specific aims were the core of the grant. Specific Aim 1 was essentially focused on developing the mouse models and reagents to answer the critical questions of the other specific aims. In retrospective, it is clear that our model was not adequate to answer those questions. But this is the nature of the DOD grants, to focus on projects with higher risks which have not been traditionally funded by other agencies. You don't need a reviewer to tell you that the progress of this proposal was very limited. I recognize this up front, but it does not mean that hard work was not performed. It was a frustrating experience because we have great hopes for this proposal. I only hope that a new investigator with new and better reagents may address this problem in the future.

I have made only minor changes to the report presented in 2005.

INTRODUCTION

The purpose of this research is to investigate cell lineages in the prostate gland of the mouse and to identify the cellular origin of prostate adenocarcinomas. The prostate epithelium contains, among others, luminal and basal cells. It is believed, but not proven, that a subpopulation of the basal cells may be stem cells (*1*) and that basal cells differentiate into luminal cells (*2, 3*). On the other hand, prostate cancer cells have characteristics of luminal cells (*4*). This project aims to use Cre/loxP technology (Sauer, 1998) to develop mice in which different prostate cell subpopulations are permanently labeled. Hereto, mice have been generated that express, constitutively or upon tamoxifen treatment, Cre recombinase under the control of keratin 5 (K5) or K14 promoters (active in basal cells (*6*)) or the probasin (PB) promoter (active in luminal cells of adult male mice (*6*). These mice have been crossed with ROSA26 mice (*7*), which harbor a universal promoter driving a β-galactosidase (β-gal) reporter gene preceded by a floxed stop sequence. In the resulting litter, Cre-mediated recombination is expected to remove the stop sequence and lead to permanent labeling of a cell subpopulation with β-gal.

BODY

Statement of Work – Specific Aim 1

The mice described in Specific Aim 1 of the approved Statement of Work (SOW), bigenic (K5-CreER^{T2} x ROSA26) mice and bigenic (PB-Cre x ROSA26) mice, were generated and tested. As a backup for the bigenic (K5-CreER^{T2} x ROSA26) mice, we also generated and tested bigenic (K14-CreER^{T2} x ROSA26) mice.

Generating and Testing Bigenic (K5-CreER^{T2} x ROSA26) and Bigenic (K14-CreER^{T2} x ROSA26) Mice

Three lines of the parental strain K5-CreER^{T2} were developed using reagents from Dr. P. Chambon's laboratory. Dr. Chambon also provided the parental strain K14-CreER^{T2} (*8*). The third parental strain, ROSA26 (*7*), was obtained from the M. D. Anderson Animal Facility in Houston with permission from Dr. P. Soriano. The parental strains K5-CreER^{T2} and K14-CreER^{T2} were each crossed with ROSA26. Tail DNA was isolated from the progeny and tested for the presence of Cre and β-gal by PCR analysis.

Five bigenic (K14-CreER^{T2} x ROSA26) mice and two wild type control animals were treated with 4-hydroxytamoxifen (4-OHT; 1 mg in 200 μl corn oil) intraperitoneally (i.p.) daily for 5 days and were sacrificed 48 hours after the last treatment. We collected dorsal skin (fixed in formalin, frozen in Tissue-Tek® OCT™ Compound (Miles Scientific, Naperville, IL), and snap frozen), heads (fixed in formalin), thymus (formalin and snap frozen), and genitourinary tract (fixed in formalin). Immunohistochemical analysis of β-gal was performed with an anti-β-gal polyclonal antibody (AB1211; 1:1000 dilution; Chemicon, Temecula, CA) in formalin-fixed tissues. We found no staining in the prostate luminal cells and inconclusive staining in the prostate basal cells. The β-gal in the bigenic mice has no nuclear location signal and thus resides in the cytoplasm. The inconclusive staining for β-gal in the prostate basal cells may have been due to the little amount of cytoplasm in these cells.

Five mice of each of the three lines of bigenic (K5-CreER^{T2} x ROSA26) mice were treated with 4-OHT (1 mg in 200 μl corn oil i.p. daily for 5 days) and sacrificed 48 hours after the last treatment. Dorsal skin (fixed in formalin, frozen in Tissue-Tek® OCT™ Compound, and snap frozen), heads (fixed in formalin), thymus (formalin and snap frozen), and genitourinary tract (fixed in formalin) were collected and analyzed for the presence of β-gal by immunohistochemistry. We detected β-gal in the skin but saw marginal reaction in the prostate. Therefore, mice of the bigenic (K5-CreER^{T2} x ROSA26) line with the highest Cre expression in the skin were treated with 4-(1 mg in 200 μl corn oil i.p. daily for 5 days) and sacrificed 48 hours after the last treatment. We did not detect β gal in the prostates of these animals.

Next, we looked into two possible causes for the absence of β gal in the prostate basal cells of the bigenic (K5-CreER^{T2} x ROSA26) mice: lack of recombination (the K5 promoter may not be strong enough in the prostate to induce a critical level of Cre), or low sensitivity of the β gal detection method.

The possibility of lack of recombination was tested by immunohistochemical analysis of formalin-fixed skin, thymus, and urogenital tissues with a polyclonal anti-Cre antibody (69050-3; 1:3000; Novagen, Madison, WI). K5-Cre mice, which constitutively express Cre, served as a positive control. Nuclei in the skin but not the bladder of K5-Cre mice reacted positively with the anti-Cre antibody. No reaction with the anti-Cre antibody was observed in the nuclei of skin, thymus, and bladder collected from K5-CreER^{T2} mice that received 4-OHT i.p. (1 mg in 200 μl corn oil daily for 5 days). In contrast, nuclei in the skin of K5-CreER^{T2} mice that received a daily topical treatment of 1 mg 4-OHT for 5 days were positive for Cre.

We used immunofluorescence as a possibly more sensitive method to detect binding of an anti-β-gal antibody (AB1211; 1:1000 dilution; Chemicon) to formalin-fixed prostate tissue. This technique did not work: the positive controls were negative. In light of the immunohistochemical results with the anti-Cre antibody, we concluded that the Cre levels induced by the K5 promoter in the prostate basal cells are probably too low to produce recombination of the β-gal gene.

Generating and Testing Bigenic (PB-Cre x ROSA26) Mice

The parental strain PB-Cre, which was originally developed at the University of Southern California (*9*), was established as a colony and crossed with ROSA26 mice. Tail DNA was isolated from the progeny and tested for the presence of Cre and β-gal by PCR analysis.

Urogenital tract was collected from four bigenic (PB-Cre x ROSA26) mice and two wild type animals were subjected to immunohistochemical analysis with an anti-β-gal antibody (AB1211; 1:1000 dilution; Chemicon). We obtained a clear, positive signal in the prostate luminal cells in the four bigenic mice and, as expected, no signal in the wild type animals. We did not detect β-gal in the prostate basal cells.

Statement of Work – Specific Aims 2 and 3

Work related to Specific Aims 2 and 3 has not been carried out. As discussed above the K5 promoter is too weak to generate efficient recombination in the Rosa 26 mice and therefore, it was not possible to pursue the most important question, i.e., the involvement of basal cells in the development of prostate cancer. In the revised SOW, we limited the studies to the Probasin-Cre mice, which will indicate whether tumors are arising from the luminal cells. We have confirmed that in our hands, the Probasin-Cre transgene, is able to recombine the Rosa 26 gene and generate b-gal activity in the luminal cells (see previous section). Unfortunately, these experiments have been delayed because our colony of Probasin-Cre mice was eliminated due to a miscommunication during the reorganization of our vivarium. The Probasin-Cre mice were reordered from the NIH repository and after delays due to issues with MTAs and health certificates; they were finally received on May 23th. Therefore, the proposed experiments were not finished in this period.

KEY RESEARCH ACCOMPLISHMENTS

- Performed all mouse crosses needed for Specific Aim 1 of the approved SOW and collected tissue samples for β-gal analysis.
- In K5-Cre mice, expression of Cre can be detected immunohistochemically only in basal cells of the skin, not in prostate basal cells.

- In K5-CreER^{T2} mice, Cre expression is induced by topical but not intraperitoneal administration of tamoxifen.
- In bigenic (PB-Cre x ROSA26) mice, β-gal is detected by immunohistochemistry in prostate luminal cells, not prostate basal cells.

REPORTABLE OUTCOMES

We developed the following animal models:

- K5-Cre mice – which carry Cre recombinase under control of the K5 promoter.
- ROSA26 mice (homozygous) – that have been developed from Dr. Soriano's—heterozygous—ROSA26 mice and carry, under control of an universal promoter, a β-gal reporter gene preceded by a floxed stop sequence.
- K5-CreER^{T2} mice – which carry a construct of the Cre recombinase and a mutated estrogen receptor under control of the K5 promoter.
- K14-CreER^{T2} mice– which carry a construct of the Cre recombinase and a mutated estrogen receptor under control of the K14 promoter.
- Bigenic (K5-CreER^{T2} x ROSA26) mice – which carry a construct of the Cre recombinase and a mutated estrogen receptor under control of the K5 promoter and a β-gal reporter gene under control of a universal promoter.
- Bigenic (K14-CreER^{T2} x ROSA26) mice – which carry a construct of the Cre recombinase and a mutated estrogen receptor under control of the K14 promoter and, under control of a universal promoter, a β-gal reporter gene preceded by a floxed stop sequence.
- Bigenic (PB-Cre x ROSA26) mice which harbor Cre recombinase under control of the PB promoter and, under control of a universal promoter, a β-gal reporter gene preceded by a floxed stop sequence.

CONCLUSIONS

We generated and tested the lines needed for Specific Aim 1 of the approved SOW, bigenic (K5-CreER^{T2} x ROSA26) mice and bigenic (PB-Cre x ROSA26) mice. As a backup, we also tested bigenic (K14-CreER^{T2} x ROSA26) mice. Immunohistochemical analysis did not detect β-gal in the prostate basal cells of the K5 and K14 bigenic lines. We also carried out experiments using K5-Cre mice. Unlike the K5-CreER^{T2} Cre is constitutively activated in the K5-Cre mice and therefore, no treatment with 4-OHT is necessary. Immunohistochemical analysis showed that staining for Cre was detectable in the skin but not in the bladder or prostate of K5-Cre mice. In a previous experiment we had shown that bigenic (K5-Cre x ROSA26) mice induce recombination in skin but not in prostate. These experiments indicate that lack of recombination is not occurring only due to low levels of 4-OHT in the prostate after systemic administration but show that promoter from keratins of the prostate basal cells are too weak to generate a critical level of Cre. However, levels of 4-OHT in the target organ may also be a limiting factor as shown by another experiment carried out in the skin of K5-CreER^{T2} mice. In this experiment we compared topical vs. systemic administration of 4-OHT and we detected Cre immunohistochemically after topical but not intraperitoneal administration. Taken together, these experiments showed that the lack of β-gal recombination in our bitransgenic mice is the result of both weakness of the keratin promoter and lack of adequate levels of 4-OHT. Therefore, new mouse models will be necessary before these experiments can be accomplished.

In bigenic (PB-Cre x ROSA26) mice, we detected β-gal by immunohistochemistry in prostate luminal cells, not prostate basal cells and therefore these mice will be a valuable model to study the fate of luminal cells during development of the prostate.

The knowledge obtained by the PI is of value to the scientific community: his findings that i.p. Tamoxifen does not induce Cre expression in bigenic (K5-CreER^{T2} x ROSA26) mice and that the K5 promoter is unsuitable for directing gene expression and recombination to the prostate basal cells were unexpected based on the available literature. Furthermore, the experience gained by the PI's laboratory has been valuable in the development of other projects focused on prostate cancer.

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